Indian J. Phys. **72B** (2), 165-169 (1998)

IJP B
an international journal

Enzyme crystal structure in an organic solvent

Sujata Sharma and Tej Singh

Department of Biophysics, All India Institute of Medical Sciences, New Delhi-110 029, India

Received 13 January 1998, accepted 24 February 1998

Abstract The crystals of proteinase *K* were grown in 80% ethanol The structure of the enzyme in this organic solvent shows considerable differences when compared with the structure of the native enzyme The protein segments of recognition site and the segments surrounded by high solvent regions show particularly large structural differences The rest of the molecule has been found to be relatively unaffected The most significant observation pertains to the relatively large perturbations of the active site residues thus making the enzyme inactive The enzyme is active again after it is placed in the aqueous medium This reversible inactivity of the enzyme in ethanol can be exploited to enhance the shelf life of the protein. These results indicate a potential application of organic solvents as preservatives of the enzymes

Keywords . Proteinase *K*, organic solvent structure,

PACS No. . 87 15 By

1. Introduction

The scope of enzyme structure, functions and stability in organic solvents is widening rapidly. The structure of enzyme in such a milieu should aid in the understanding and optimization of its function. It has become clear from the recent vigorous studies in nonaqueous enzymology that enzymes are not only stable in organic solvents but also exhibit different functional properties [1-3]. To understand the role of organic solvents and various phenomena associated with it, it is critical to elucidate protein conformation in organic media and compare it with the same in aqueous solution. In view of this, we have determined the crystal structure of proteinase *K* in ethanol.

2. Experimental

Proteinase *K* was obtained from Sigma Chem. Co. (USA). It was purified by gel filtration on a Sephadex G-75 column in 50 mM Tris. HCl, pH 7.5 containing 1mM $CaCl_2$. Fractions of highest activity were pooled, dialyzed exhaustively at 4°C against 1 mM calcium acetate and lyophilized. The lyophilized powder was dissolved in 0.01 M Tris. HCl, pH 8.0 to a final concentration of 10 mg/ml. This was dialyzed against the same buffer with 80% ethanol (v/v) at 4°C. The crystals

grew slowly in a period of 3-4 months. The typical dimensions of square pyramidal crystals were of the order of 0.2 x 0.15 x 0.1 mm^3. Although the crystals were relatively small, they diffracted well upto 3.0 Å resolution. The diffraction data were collected on a MAR Research Imaging plate scanner using a graphite monochromated CuK_α radiation generated by a rotating anode generator (Rigaku : RU-200), operating at 100 mA, 40 kV with a focal point of 0.3 x 3 mm^2 Each data image covered a rotation angle of 1.0° and was exposed for 15 minutes. The intensity data were processed using DENZO and SCALEPACK program packages [4-5]. The results from the data collection are given in Table 1.

Table 1. Data collection statistics.

Space group	Tetragonal $P4_32_12$
Cell dimensions (Å)	a = b = 68 3, c = 108 3
X-ray source	Rotating anode generator, operating at 40 kV, 100 mA (focal size 0 3 x 3 mm^2)
Radiation (Å)	CuK_α /graphite monochromator, 1 54182
Collimation (mm^2)	0.3 x 0.3
Detector	MAR research imaging plate scanner
Crystal to plate distance (mm)	
Resolution (Å)	3.4
Total number of observed reflections	2141
Completeness of data upto 3.4 Å (%)	55
Rmerge (%)	6.1

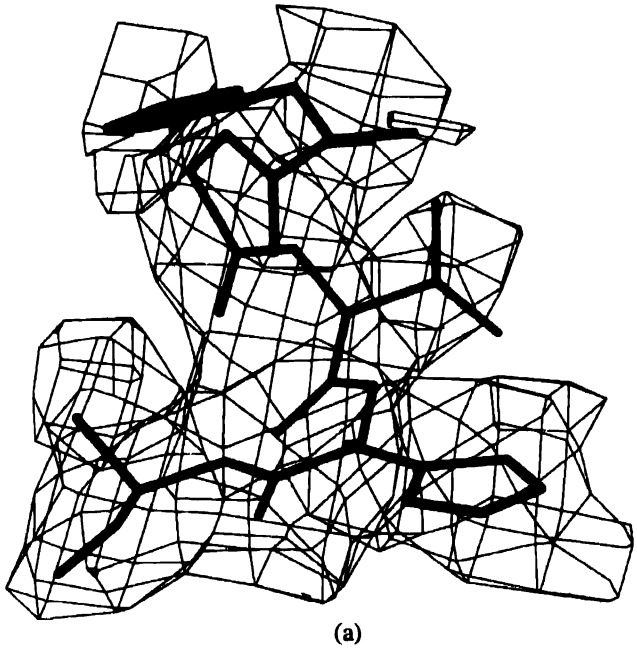
3. Structure solution and refinement

The structure was determined by molecular replacement using a model of proteinase K [6]. The structure was refined by a combined iterative procedure that included model rebuilding with the software O [7]. The refinement was performed by restrained parameter least-squares analysis using PROLSQ / PROTIN program [8] with fast Fourier routines to compute structure factors and gradient [9]. During the refinement, adjustment of side chains was performed by inspecting (2Fo-Fc) and (Fo-Fc) difference Fourier syntheses. All the graphics work was performed using a program package O [7] on an INDIGO silicon graphics system. Finally, the model was refined using stereochemically restrained least-squares procedure to an *R*-factor of 16.0 % for all the

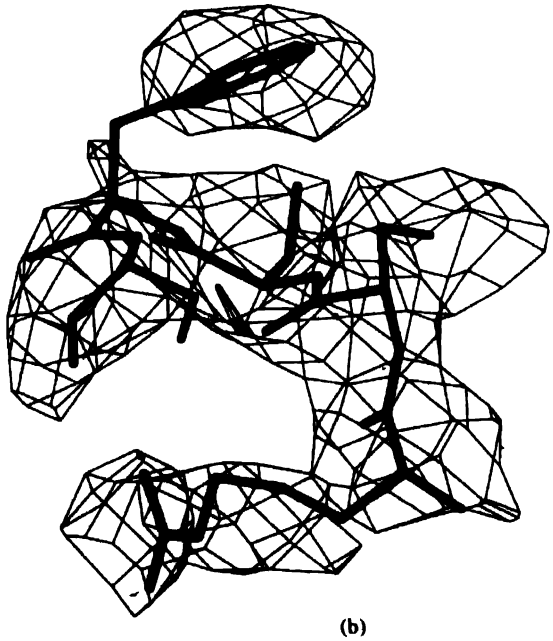
Table 2. Summary of refinement statistics

Resolution limits (Å)	17.0–3.4	
Protein atoms	2018	
R-factor (%)	16.0	
$B_{average}$ ($Å^2$)	23.5	
Overall *G*-factor	–0 45	
Stereochemistry	Target	Estimated
Bond Length (1–2) (Å)	0.020	0.024
Angle distance (1–3) (Å)	0.040	0.046
Planar distance (1–4) (Å)	0.050	0.068
Chiral volumes ($Å^3$)	0.15	0.21
Planar torsion angle (°)	5.0	5 7
Deviation from planes	0.020	0.030

data. Refinement statistics are summarized in Table 2. Two sections of electron density randomly selected from the (2Fo-Fc) map are shown in Figure 1. The refined coordinates will be deposited in the Brookhaven Data Bank.



(a)



(b)

Figure 1. Two representative regions of the electron density. Contour level is at 1.2σ

4. Results and discussion

The model contains 2018 protein atoms and one calcium ion (Figure 2). The average temperature factor is 23.5 $Å^2$. Though the model could not be refined rigorously due to the limitation of data, it showed significant features. The present structure shows large differences in certain parts of the molecule when compared with the native crystal structure [6]. As it is the first structure of proteinase *K* grown from such a buffer containing a high concentration of ethanol (80 %), it is of great interest to understand the role of organic solvent. It reveals substantial structural and functional differences as compared to the native structure of the enzyme.

Figure 2. Cα tracing of the molecule

Superposition of the refined coordinates of the proteinase *K* chain from the present model on the native structure of proteinase *K* [6] gives a root mean square discrepancy of 2.0 Å for the Cα atoms (Figure 3) and 2.8 Å for all the other atoms (Figure 4). This is to be compared with the estimated errors in the coordinates of the native (0.14 Å) and the present molecule (0.30 Å). In certain parts of the polypeptide chain, there are large differences in the Cα positions between the native enzyme and the enzyme in ethanol. The root mean square deviation of the active site residues between the native enzyme and the present structure after refinement is approximately 2.1 Å (Figure 5). There are several residues on the surface which have moved away from the corresponding residues in the native structure by more than Cα displacements 3.5 Å.

The substrate recognition site is formed by two peptide chains Asn 99-Tyr 104 and Ser 132-Gly 136. The major conformational changes occur in these regions of activation domain. The segments Asn 99 to Tyr 104 and Ser 132 to Gly 136 adopt significantly different

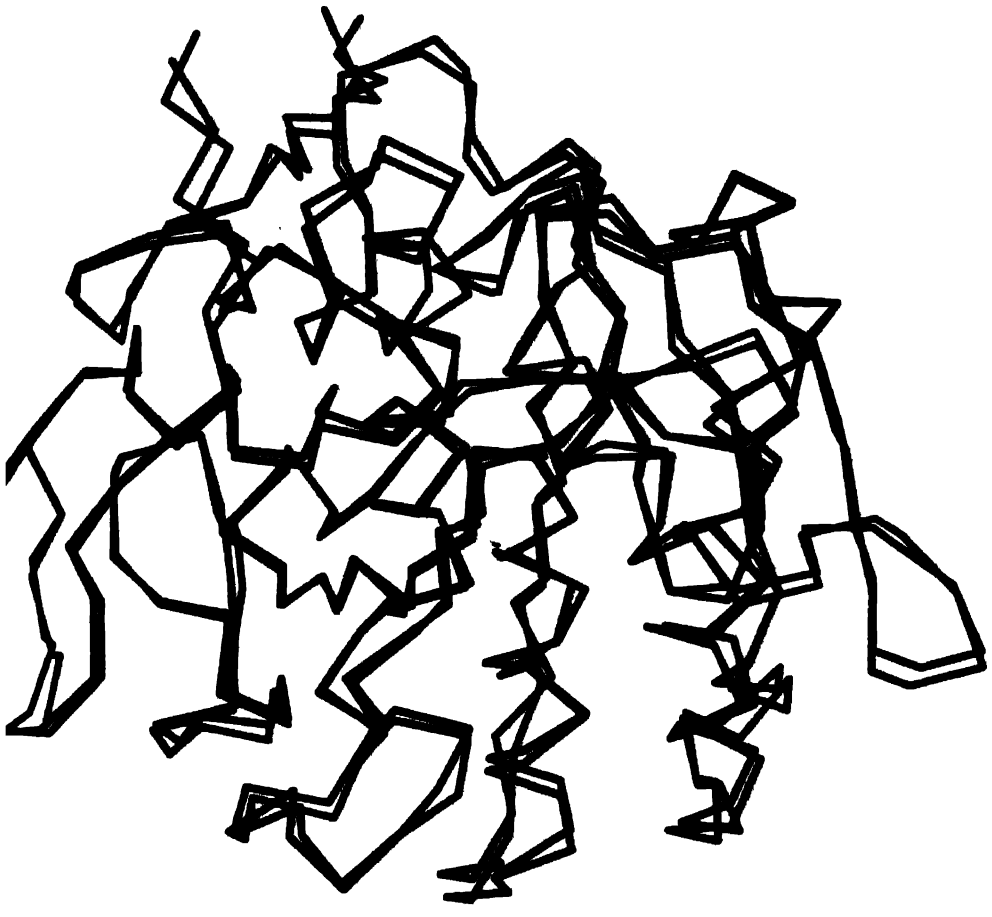


Figure 3. Superposition of the backbone : proteinase *K* in ethanol (red) and native proteinase *K* (blue).

conform
the rec
proxin
in wat

Plate II

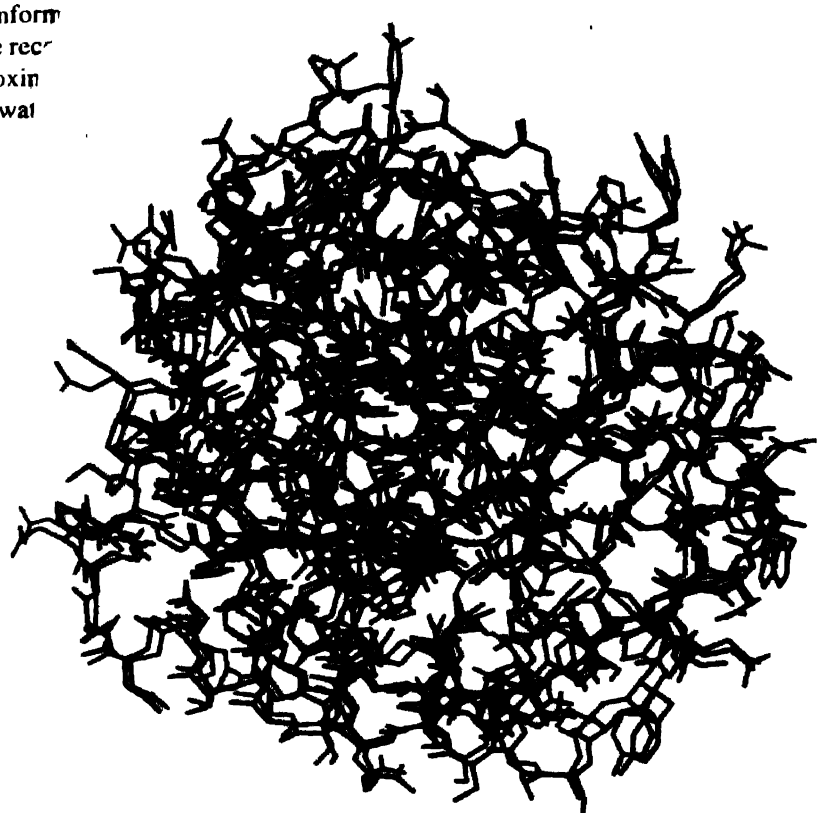


Figure 4. Superposition of the complete molecule : Proteinase *K* in ethanol (red) and native proteinase *K* (green)

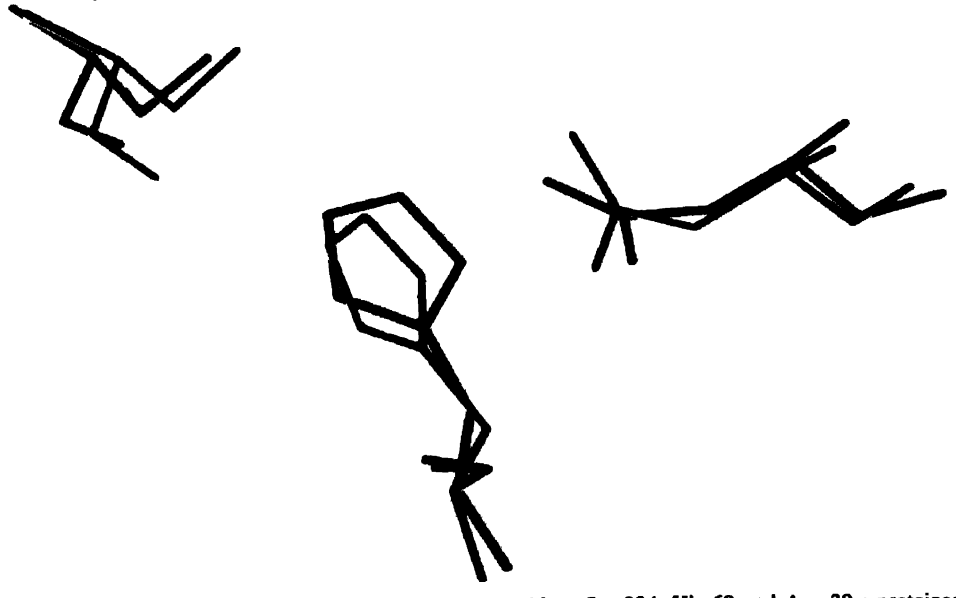


Figure 5. Superposition of the active site residues Ser 224, His 69 and Asp 39 : proteinase *K* in ethanol (red) and native proteinase *K* (green).

conformations when compared with the native structure. It suggests that the two strands of the recognition site are very flexible parts of the protein. In addition to it, the segments in the proximity of the *N*-terminus also show large scale conformational changes. This region is rich in water contents and hence gets affected considerably.

The calcium positions are also substantially influenced. The Ca^{2+} which is located in the proximity of Pro 175 remains well-coordinated and the coordinating bonds are only slightly elongated whereas the Ca^{2+} in the region of Thr16 seems to have been lost into the ethanol environment as it could not be observed in the difference electron density map.

Therefore, the results of these investigations clearly indicate that the introduction of ethanol into proteinase *K* crystals causes large scale structural changes. In this case, the distance between Ser 224 O^{γ} to His 69 $N^{\varepsilon 2}$ is 4.19 Å. The overall effect of ethanol on the enzyme results in the inactivation of the molecule due to large scale perturbations, thus interrupting the proton relay mechanism in the enzyme.

References

[1] P A Fitzpatrick, A C U Steinmetz, D Ringe and A M Klibanov *Proc Natl Acad Sci (USA)* **90** 8653 (1993)

[2] J Travin *Science* **262** 1374 (1993)

[3] J L Schmitke, L J Stern and A M Klibanov *Proc Natl Acad. Sci (USA)* **94** 4250 (1997)

[4] Z Otwonowki *Oscillation Data Reduction Program . in Proceedings of the CCP4 Study Weekend data Collection and Processing, (29-30 January 1993)* eds L Sawyer, N Isaacs and S Bailey (56-62 Warrington · Daresbury Laboratory) (1993)

[5] W Minor *XDISPLAYEF Program* (Purdue University, West Lafayette, USA) (1993)

[6] Ch Betzel, G P Pal and W Saenger *Eur J Biochem.* **178** (1988)

[7] T A Jones, Y Zou, S W Cowan and M Kjeldgaard *Acta Crystallogr* **A47** 110 (1985)

[8] W A Hendricksson and J K Konnert in *Biomolecular Structure, Function, Conformation and Evolution* ed. R Srinivasan (Oxford Pergamon) **Vol 1** p 43 (1981)

[9] R C Agarwal *Acta Crystallogr.* **A34** 791 (1978)